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基于形态和分子证据的大花忍冬复合群的种间关系

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摘要: 大花忍冬复合群主要分布在中国南部及周边地区, 是忍冬属的主要疑难类群之一, 包含大花忍冬、灰毡毛忍冬、细毡毛忍冬、菰腺忍冬和锈毛忍冬5种。该复合群是中国南部金银花的重要野生替代资源。本研究通过形态和生境观察、叶表皮微形态扫描和分子系统学分析相结合的方法, 探讨了大花忍冬复合群的种间关系。结果表明, 分子系统学方法没有解决该复合群之间的关系, 仅灰毡毛忍冬的区分较为明显, 基于形态学证据大致可将大花忍冬复合群分为以下3小群: 菰腺忍冬以其独有的蘑菇状腺体与其他4种显著不同, 毛被长度也明显长于其他种类; 锈毛忍冬与大花忍冬的毛被较稀疏, 但锈毛忍冬的花冠筒较短, 气孔特征独特; 灰毡毛忍冬与细毡毛忍冬的叶背密覆柔毛, 二者的上表皮角质膜结构有差异。该复合群的物种形成和传播机制仍待深入研究。

关键词: 忍冬属; 复合群; 叶表皮; 分子系统学; 分类
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Relationships within the *Lonicera macrantha* Complex Based on Morphological and Molecular Data

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Abstract: Being one of the most complicated groups in *Lonicera* (Caprifoliaceae) and distributed mainly in southern China, the *Lonicera macrantha* complex comprises five species, i. e. *L. macrantha*, *L. macranthoides*, *L. similis*, *L. hypoglauca* and *L. ferruginea*. The complex is also found to be the main substitute of *Ljaponica* for *Jin Yin Hua* in southern China. To clarify the relationships within the complex, investigation of morphology and habitat of each species, observation of microfeatures of leaf epidermis, and molecular phylogenetic analysis were carried out. The results show that only *L. macranthoides* can be easily distinguished by phylogenetic methods, and that the complex can be roughly divided into three groups based on morphological characters: *L. hypoglauca* is distinct in its mushroom-shaped glands and long trichomes on leaf epidermis; *L. ferruginea* is close to *L. macrantha* in the thin trichomes, but they differ in length of corolla tube and stomatal structure; *L. macranthoides* is morphologically similar to *L. similis* in the dense trichomes, but they can be distinguished by characters of cuticular membrane. Further studies on resolving the speciation and species dispersal within the complex are still needed.

Key words: *Lonicera*; Species complex; Epidermis; Molecular phylogeny; Taxonomy

A great deal of molecular phylogenetic studies suggest that Caprifoliaceae *s. l.* is not monophyletic, but should be separated into several small families (Donoghue *et al.*, 1992, 2001a, b, 2003; Angio-

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sperm Phylogeny Group, 1998; Kim and Kim, 1999; Pyck *et al.*, 1999; Zhang *et al.*, 2003; Jacobs *et al.*, 2010; Tank and Donoghue, 2010; Howarth *et al.*, 2011; Landrein *et al.*, 2012) or expanded to a much bigger family combining with Dipsacaceae Juss., Valerianaceae Batsch and Morinaceae Raf. (Judd *et al.*, 2007; Mabberley, 2008; Bremer *et al.*, 2009). Caprifoliaceae *s. s.* consists of *Lonicera* Linn. and four small genera, i. e. *Leycesteria* Wall., *Triosteum* Linn., *Heptacodium* Rehder and *Symphoricarpos* Duhamel (Yang *et al.*, 2011).

The genus *Lonicera* contains about 180 species distributed in North Africa, Asia, Europe and North America, of which 57 can be found in China (Yang *et al.*, 2011). Traditionally, *Lonicera* was dvided into subgen. *Lonicera* (*ca.* 155 species) and subgen. *Caprifolium* (*ca.* 25 species) according to morphology of flowers (paired or not) and top leaves (associated or not) (Rehder, 1903). *S*ubgenus *Lonicera* was further separated into four sections, i. e. sects. *Isoxylosteum* Rehd., *Isika* DC. ex Rehd., *Coeloxylosteum* Rehd. and *Nintooa* DC., according to their habitat, branches (hollow or solid) and corolla tubes (gibbous or not at the base). The two subgenera were supported by phylogenetic analyses, but not with the sections and subsections (Theis *et al.*, 2008; Smith, 2009). For example, the two subsections of *Lonicera* sect. *Nintooa* were embedded within different clades (Theis *et al.*, 2008).

The *Lonicera macrantha* complex (*Lonicera* sect. *Nintooa*), is one of the most complicated groups in genus *Lonicera*. Five species are included within the complex, i. e. *L. macrantha* (D. Don) Spreng, *L. macranthoides* Hand. -Mazz., *L. similis* Hemsl., *L. hypoglauca* Miq. and *L. ferruginea* Rehd., sharing similar floral structure, various leaf shapes, habitat and sympatric distribution. They are distributed in southern China and adjacent area, and mainly grow at the edge of forests or roadside. Two obvious distribution centers, Nanling and Wuling Mountains, of *L. hypoglauca* and *L. similis*, respectively, have been recognized. All the five species are large woody climbers with hollow branches and 2-lipped corolla, and their buds are made into the traditional Chinese medicine *Jin Yin Hua*. They can be distinguished from each other mainly by trichomes on abaxial leaf epidermis.

To clarify the relationships among the five species of the complex and further contribute to a better use of them, investigation of morphology and habitat of each species, observation of microfeatures of leaf epidermis, and molecular phylogenetic analysis were carried out in this study.

1 Materials and methods

1.1 Materials

Materials of all five species of *L. macrantha* complex and five other close taxa from *Lonicera* sect. *Nintooa* [*L. calcarata* Hemsl., *L. nubium* (Hand.-Mazz.) Hand.-Mazz., *L. confus* DC., *L. pampaninii* H. Lévl. and *L. japonica* Thunb.] were collected from Guangdong, Guangxi, Guizhou, Jiangxi, Sichuan, Yunnan and Zhejiang Province, China. In total of 27 populations and 81 individuals were included in this study with 1−5 individuals in each population. *Sambucus chinensis* Lindl. was selected as outgroup in molecular phylogenetic analysis. Voucher information of these taxa is listed in the Appendix.

1.2 Epidermal observation

Micromorphology of leaf epidermis was investigated using scanning electron microscopy (SEM). Leaves from the five species of *L. macrantha* complex at various stages of development were fixed for 2 h at 41 ℃ with 2% glutaraldehyde in 0.1 mol·L^{-1} sodium cacodylate buffer (pH = 7.2). After washing with the same buffer and dehydrating in acetone, the material was mounted onto stubs, and coated with gold-palladium (Ascensao *et al.*, 1997). Observations were conducted using a Hitachi-S4800 scanning electronic microscope (Hitachi Ltd., Tokyo, Japan) with 10 kV voltage.

1.3 DNA extraction, PCR amplification and sequencing

Total DNA was extracted from silica-gel-dried leaves using a modified CTAB extraction protocol

(Doyle and Doyle, 1987). Extracts were used as template for amplification of two plastid DNA regions (*trn*S-*trn*G intergenic spacer and *psb*A-*trn*H intergenic region).

Polymerase chain reaction (PCR) amplification of *trn*S-*trn*G region was performed with primers *trn*S (GSU) and *trn*G (UCC) (Hamilton, 1999), while that of *psb*A-*trn*H was carried out using primers *psb*A and *trn*H (Hamilton, 1999). The 25 μL volume reaction mixtures contained 2 μL of sample DNA, 0.3 μL of *Taq* DNA polymerase (Tiangen Biotech Co., Ltd., Beijing, China), 2.5 μL of 10 × reaction buffer, 1.5 μL of $MgCl_2$, 2.5 μL of dNTPs, 1 μL of bovine serum albumin (BSA, 20 mg · mL^{-1}) and 0.5 μL of each primer. Amplifications were performed using a program consisting of an initial denaturation at 80 ℃ for 5 min followed by 30 cycles of 45 s denaturation (94 ℃), 45 s annealing (52 ℃) and 50 s extension (65 ℃), ending with a final extension at 65 ℃ for 7 min. The PCR reaction system and amplification protocol were identical for above two regions.

PCR amplifications were performed with a Biometra T1 thermocycler (Biometra, Göttingen, Germany). The amplified products were checked on 1% TAE agarose gels and sequenced by an ABI-PRISM3730 sequencer after purification in Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). The same primers described above for PCR amplifications were also used for the sequencing reactions.

Sequences were edited and assembled using Seqman (DNASTAR, Madison, WI, USA), and aligned by MEGA5.05 (Tamura *et al.*, 2011). For each individual, two fragments (forward and reverse) were aligned to obtain a full-length sequence for each region. Poly-A/T index that seemed to be caused by DNA polymerase error was removed from the dataset. All other indices were coded manually using the simple indel coding method of Simmons and Ochoterena (2000) as binary characters.

1.4 Phylogenetic analyses

1.4.1 *Maximum Parsimony (MP) analysis*

The combined *trn*S-*trn*G and *psb*A-*trn*H dataset was analyzed under a maximum parsimony criterion implemented by PAUP* version 4.0b10 (Swofford, 2002). Only one sequence representing each haplotype was used in the phylogenetic analysis. Heuristic searches were performed with 100 replicates of random stepwise additions, holding 10 trees at each step with tree-bisection-reconnection (TBR) branch swapping and unlimited MaxTrees. Bootstrap support value (BS) was estimated with 100 bootstrap replicates using full heuristic search with TBR and Multrees in effect.

1.4.2 *Bayesian Inference (BI) analysis*

The model GTR+I+G was chosen as the best fit for the combined dataset by Modeltest 3.6 (Posada and Crandall, 1998). Based on the model selected, the dataset was analyzed using MrBayes 3.2.1 (Ronquist and Huelsenbeck, 2003). Markov chain Monte Carlo (MCMC) iterations were carried out with four chains (one cold, three heated) for 10 000 000 generations, sampling a tree every 1 000 generations. Convergence of runs was accepted when the standard deviation of split frequencies (SDSF) fell below 0.01. The first 25% of the sampled generations were discarded as burn-in and the posterior probabilities (PP) values were determined from the remaining trees.

2 Results

2.1 Morphological data

The main morphological characters of the complex are listed in Table 1. The leaf texture of *L. macrantha* and *L. macranthoides* is leathery, while that of other three is papery. The inflorescences of two species are corymbose and cone-shaped, while those of *L. similis*, *L. hypoglauca* and *L. ferruginea* are short racemose to racemose. Proportion of length of corolla tube and lip in *L. macrantha* is much higher than that of the others. Length of corolla of *L. ferruginea* is short, while bract length of which is long, and that of the rest four species are similar. The peduncle length of *L. similis* is much longer than that of other four taxa. Fruits of all five species are black to

blue-black, but only those of *L. macranthoides* and *L. hypoglauca* have powder on the surface.

Most of the characters are quantitative, but there is no obvious gap among them. The most significant difference between the five species is the type of trichome on abaxial leaf epidermis. Therefore we observed the trichomes using SEM in order to get more detailed informative (Table 2 and Fig. 1). At the same time, characteristics of cuticular membrane and stomata were also observed. It has been proved that trichomes can be used for identification of some species and medicine materials (Huang and Chen, 2005), and may be results of adaption to different habitats (Li *et al.*, 2007).

2.2 Micromorphological data

2.2.1 *Cuticular ornamentation*

Only the cuticula of *L. macrantha* is nearly smooth, others swell into different types: cuticular ornamentation of *L. similis* is striated, while that of *L. hypoglauca* and *L. ferruginea* is ridged, and irregular cuticula is found in *L. macranthoides* (Figs. 1: A, D, G, J, M).

2.2.2 *Trichome*

All the five species have single hairs on both leaf surfaces, but most trichomes appear on abaxial epidermis, except for *L. hypoglauca* and *L. ferruginea*. Hairs of *L. hypoglauca* and *L. ferruginea* are straight, but others are pubescent. Only *L. hypoglauca* has *ca.* 1 000 μm long trichomes, while that of others are 200 μm in average. Hairs of *L. macrantha*, *L. hypoglauca* and *L. ferruginea* are relatively thin, in particular of *L. hypoglauca* for the abaxial leaf surface of the species is occupied by mushroom-shaped glands instead (Fig. 1: I); trichomes of *L. macranthoides* and *L. similis* are so dense that stomata are covered and hard for observation (Figs. 1: B, E, H, K, N).

2.2.3 *Stomatal characters*

Stomatal apparatuses can only be found on abaxial surface of leaves. All the stomata observed are elliptic and 120 – 220 μm long in average with a stomatal index (number of stomata divided by number of epidermal cells per field) of 0.20. The inner margin of outer stomatal rim of all the five species is smooth. Stomata of *L. macrantha* and *L. hypoglauca* are surrounded with wax grains, while those of *L. ferruginea* are nearly clean (Figs. 1: C, F, L, O).

Table 1 Key morphological characters of the *Lonicera macrantha* complex

	L. similis	*L. macrantha*	*L. macranthoides*	*L. hypoglauca*	*L. ferruginea*
leaf texture	papery	leathery	leathery	papery	thick-papery
petiole length/mm	3–12	3–10	6–10	5–12	10
abaxial trichome	densely tomentose	strigose	densely tomentose	pubescent	brown strigose
inflorescence	racemose	corymbose	paniculate	racemose	short racemose
bract length/mm	2–4.5	2–5	2–4	3–4	6–12
peduncle length/mm	10–40	1–8	0.5–3	4–15	2–7
corolla length/cm	4–6	3.5–9	3.5–6	3.5–4	2–2.5
tube: lip	1.5:1	2.5:1	1:1	1:1	1:1
fruit color	blue-black	black	black	black	black
powder on fruit	not observed	not observed	blue-white	white	not observed

Table 2 Epidermal characters of *Lonicera macrantha* complex based on SEM

	L. similis	*L. macrantha*	*L. macranthoides*	*L. hypoglauca*	*L. ferruginea*
cuticular ornamentation	striate	smooth	irregular	ridged	ridged
abaxial trichome	pubescent	pubescent	pubescent	straight	straight
length of trichome/μm	200	200	200	1000	200
density of trichome	dense	thin	dense	sparse	thin
length of stomata/μm	not observed	220	not observed	220	120
adjunct of stomata	not observed	wax grains	not observed	wax grains	nearly clean

2.3 Molecular data

The combined matrix of *trn*S-*trn*G and *psb*A-*trn*H consist of 1 103 characters (aligned lengths were 699 for *trn*S-*trn*G and 404 for *psb*A-*trn*H), 98 of which were potentially parsimony-informative. MP analysis identified 1 000 trees [tree length = 571;

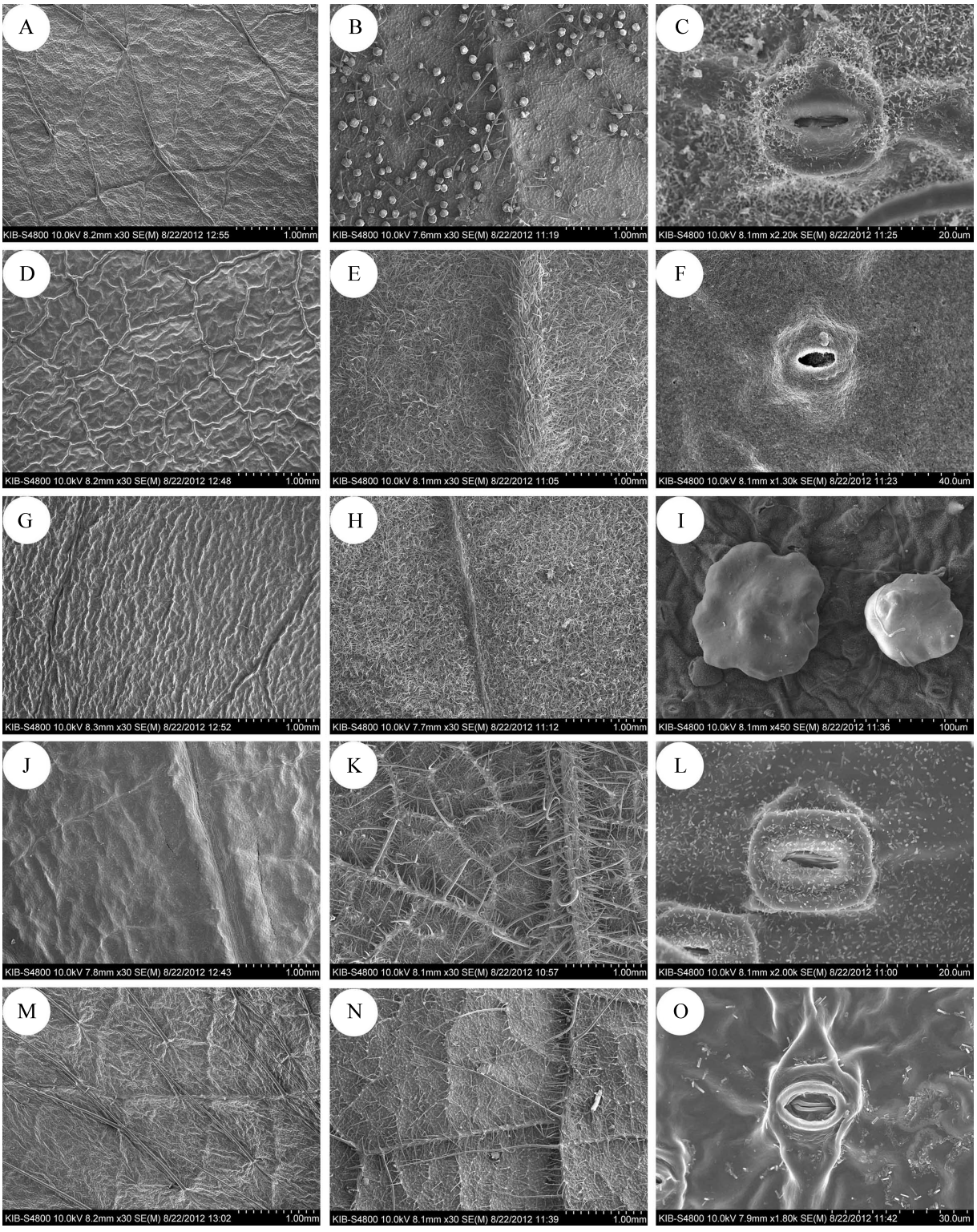

Fig. 1　Epidermal characters of *Lonicera macrantha* complex based on SEM

A–C, F, I, *L. hypoglauca*; D, E, *L. macranthoides*; G, H, *L. similis*; J–L, *L. macrantha*; M, N, O, *L. ferruginea*

consistency index (CI) = 0.855; retention index (RI) = 0.7787]. Bootstrap values (BS) greater than 50% are shown in Fig. 2, species names with population numbers are labeled at the tip of each branch. Analyses of the combined datasets using MP and BI methods yield trees with similar topology, therefore, only the MP tree is shown here with PP greater than 50% being marked after the slash of the tree (Fig. 2).

The monophyletic of the complex is only moderately supported in MP tree (BS = 56%), but is not supported by BI analysis (PP = 86%), and relationship among the five species is not resolved. All the eight individuals from two populations of the *L. macranthoides* group together (BS = 72%, PP = 99%) and show high variations within the species. But individuals of other four species of the complex can't be distinguished from each other at all from the MP tree.

3 Discussion

The five species of *Lonicera macrantha* complex are similar in morphology and habitat. Shapes and sizes of their leaves vary greatly in different growing

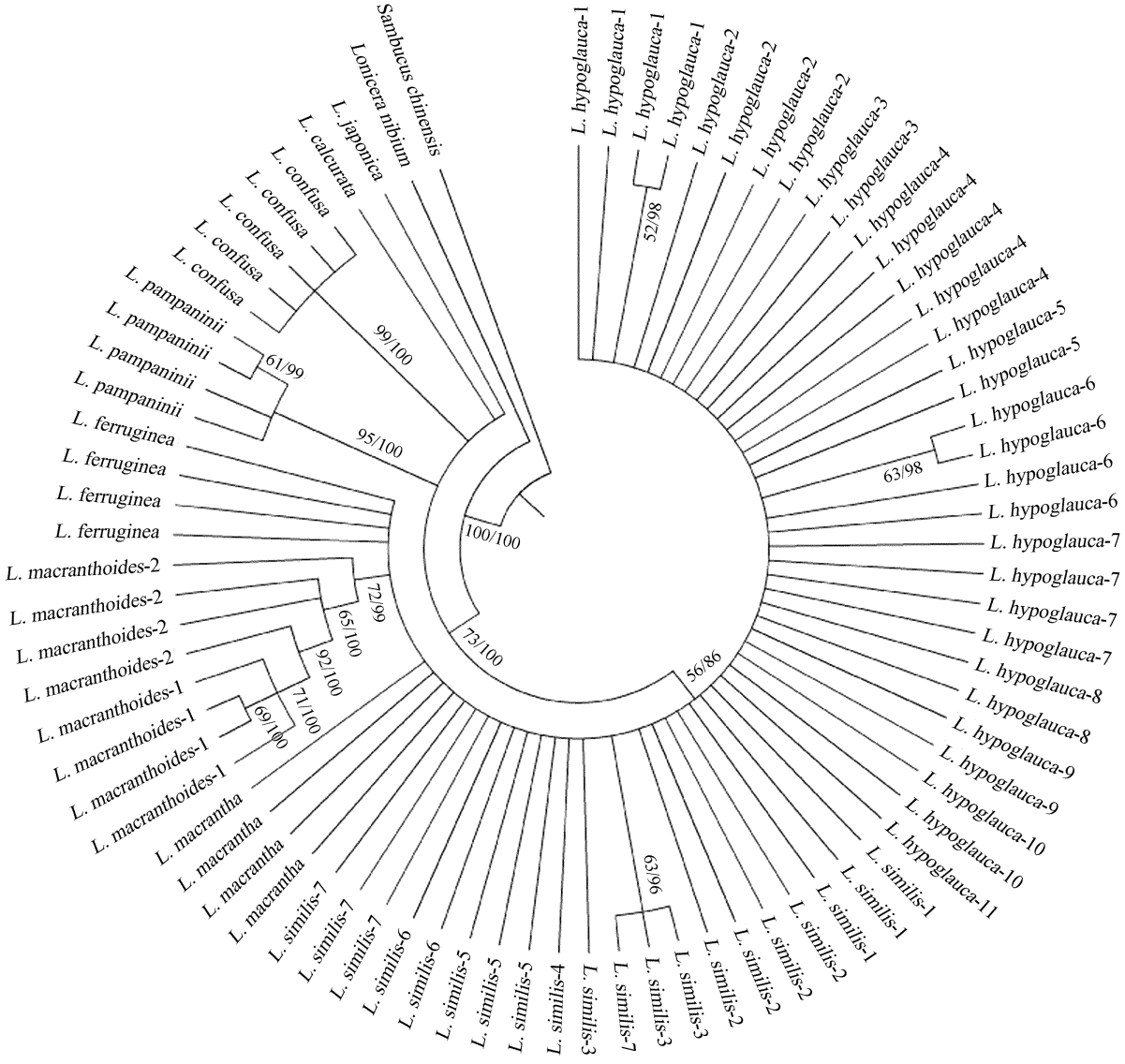

Fig. 2 Phylogram obtained from parsimony analysis based on combined plastid DNA dataset. Bootstrap values higher than 50% are indicated above branches before the slash and the Bayesian posterior probabilities higher than 50% are set after the slash

periods and habitats. After a great deal of field work and specimens examination, we find out that some of the morphological and micromorphological characters to be useful for identification of the five species, trichomes on abaxial leaf surface being the most distinct.

Currently, plastid DNA variations have been frequently used for phylogenetic studies on plants (Shaw *et al.*, 2007), for substitution rate of cpDNA is moderate for intergeneric or intrageneric phylogenetic reconstruction and suitable universal primers have been developed for many higher plants (Taberlet *et al.*, 1991; Soltis and Soltis, 1998; Shaw *et al.*, 2007). In our phylogenetic analysis, two plastid DNA regions, i. e. *psb*A-*trn*H intergenic spacer and *trn*S-*trn*G intergenic spacer, were combined to look into the relationships of the five species. Sun *et al.* (2011) used seven candidate DNA barcodes (*psb*A-*trn*H, *mat*K, *rbc*L, *trn*L intron *trn*L-*trn*F spacer, ITS2 and ITS) for identification of *L. japonica* and its related species, and their results showed that the highest interspecific divergence was provided by *psb*A-*trn*H region. The *trn*S-*trn*G intergenic spacer contributed even more variable loci than *psb*A-*trn*H in our study. However, only the eight individuals of *L. macranthoides* can be well recognized from the phylogenetic results, while individuals of other four species and relationships between the five species are still not resolved.

The explanation for this unresolved phylogeny may be incomplete lineage sorting of ancestral polymorphisms before the rapid divergence of these species, making plastid DNA sequences insufficient to resolve the phylogenetic relationships. To further look into the relationships among these species and their relationships with other related taxa of *Lonicera*, an expanded sampling within the genus as well as more available DNA markers, such as single copy nuclear genes which contain more loci, will surely be helpful for the phylogenetic reconstruction (Whittall *et al.*, 2006).

Based on morphological and micromorphological evidence, the complex could be roughly divided into three groups: *Lonicera hypoglauca* is a relatively distinctive species for its mushroom-shaped glands and extremely long trichomes on abaxial leaf epidermis; *L. macranthoides* and *L. similis* are similar to each other in character of trichomes that both of them have dense pubescent hairs on abaxial surface, but they differ in types of cuticular ornamentation; *Lonicera macrantha* and *L. ferruginea* are characterized with thin hairs on abaxial surface of leaves, while stomatal structure and corolla length of *L. ferruginea* is obviously different from that of the former.

Relationships within the *L. macrantha* complex are still obscure and complicated, simple merging or division of the five species is not suggested here. They could be carefully distinguished by morphology and micromorphology, but so far no more effective information can be required from the phylogenetic analyses. Further studies on resolving the monophyletic of the complex and speciation and spreading mechanism of this complex are still badly needed.

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Appendix: Voucher and locality of species sampled in this study (all deposited in KUN)

No.	Taxa	Locality	Voucher
1-4	*Lonicera hypoglauca*	Longlin, Guangxi	Dong H. J. & Yin Z. J. 211
5-8	*L. hypoglauca*	Nanchuang, Chongqing	Dong H. J. & Tang Y. 284
9-10	*L. hypoglauca*	Longlin, Guangxi	Dong H. J. & Yin Z. J. 210
11-15	*L. hypoglauca*	Lechang, Guangdong	Dong H. J. & Yin Z. J. 180
16-17	*L. hypoglauca*	Quanzhou, Guangxi	Dong H. J. & Yin Z. J. 205
18-21	*L. hypoglauca*	Renhua, Guangdong	Dong H. J. & Yin Z. J. 168
22-25	*L. hypoglauca*	Dayu, Jiangxi	Dong H. J. & Yin Z. J. 154
26-27	*L. hypoglauca*	Chengbu, Hunan	Dong H. J. & Yin Z. J. 184
28-29	*L. hypoglauca*	Chengbu, Hunan	Dong H. J. & Yin Z. J. 185
30-31	*L. hypoglauca*	Lichuang, Hubei	Dong H. J. & Tang Y. 259
32	*L. hypoglauca*	Jiangkou, Guizhou	Dong H. J. *et al.* 931
33-35	*L. similis*	Emei, Sichuan	Dong H. J. & Tang Y. 334-A
36-39	*L. similis*	Lichuang, Hubei	Dong H. J. & Tang Y. 258
40-42	*L. similis*	Hefeng, Hubei	Dong H. J. & Tang Y. 283
43	*L. similis*	Yuanmou, Yunnan	Fang W. 11153
44-46	*L. similis*	Yuanmou, Yunnan	Xiang C. L. *et al.* 503
47-48	*L. similis*	Wuding, Yunnan	Xiang C. L. *et al.* 511
49-52	*L. similis*	Jiangkou, Guizhou	Dong H. J. *et al.* 654
53-56	*L. macrantha*	Quanzhou, Guangxi	Dong H. J. & Yin Z. J. 204
57-60	*L. macranthoides*	Lechang, Guangdong	Dong H. J. & Yin Z. J. 178
61-64	*L. macranthoides*	Quanzhou, Guangxi	Dong H. J. & Yin Z. J. 206
65-68	*L. ferruginea*	Jingdong, Yunnan	Dong H. J. *et al.* 837
69-72	*L. pampaninii*	Lechang, Guangdong	Dong H. J. & Yin Z. J. 177
73-77	*L. confusa*	Guilin, Guangxi	Shen X. L. s. n.
78	*L. calcurata*	Xundian, Yunnan	Dong H. J. *et al.* 841
79	*L. nubium*	Emei, Sichuan	Dong H. J. & Tang Y. 334-B
80	*L. japonica*	Tianmushan, Zhejiang	Dong H. J. & Yin Z. J. 042